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14. ABSTRACT The studies examined mechanism based therapies to prevent or treat noise induced tinnitus. Our studies showed a military relevant small arms fire-like noise will induce tinnitus in approximately 33% of exposed rats. There is increasing evidence that noise induced loss of connections between sensory cells and the auditory nerve can induce tinnitus. Studies therefore examined if anti-oxidant and or anti-excitotoxicity therapy could decrease the loss of these connections from the small arms fire-like noise and if so, prevent tinnitus. Results first showed that a combination of Piribedil and Memantine (anti-excitotoxicity) plus Vitamins A, C and E plus magnesium (anti-oxidants) provided protection, with significantly reduced loss of connections. Studies next tested if this treatment would then reduce tinnitus. The results showed decreased incidence of tinnitus following noise in the group receiving this combined treatment prior to the noise compared to groups of noise exposed animals without treatment. The next set of studies targeted the auditory brain stem and treatment rather than prevention. Studies tested treatment with sarcosine, which increases inhibitory influence by decreasing uptake of glycine from synapses. The studies found that sarcosine given shortly after the noise exposure or shortly before the noise did not influence the incidence of tinnitus. However, sarcosine when provided over a 4 - 6 week period following the noise showed a trend towards inducing recovery in animals that developed tinnitus. Future studies will be needed to follow up on this finding.					
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I. INTRODUCTION:

A high incidence of tinnitus can result as an outcome of battlefield noise and this has become a major health concern for military, reducing the ability to redeploy, reducing the quality of life of those affected and increasing health care costs. These studies had two goals. One goal was “PREVENTION”, to develop treatments that if provided prior to a noise exposure will prevent tinnitus from being induced. Studies tested if loss of inner hair cell –auditory nerve connections plays an initiating role in generation of tinnitus and if preventing this loss will prevent tinnitus from occurring. Studies first tested to identify treatments to prevent loss of connections and then tested if such treatment would prevent the progression of tinnitus, resulting in decreased incidence of tinnitus in noise exposed rats receiving the treatment versus untreated noise exposed rats. A small arms fire-like noise was used for military relevance. Anti-excitotoxic agents were identified and were found to reduce the loss of connections and treatment with these agents (Piribedil and Memantine) was found to reduce the incidence of noise-induced tinnitus in treated animals. The second goal was “TREATMENT”, to develop interventions that can halt the progression of tinnitus when given after a noise exposure. Studies tested enhancing the influence of glycine, a major inhibitory transmitter to prevent increased excitability from developing in central auditory pathways. Treatment with sarcosine, which enhances the inhibitory influence of glycine, was not found to decrease the incidence of tinnitus, but generated a strong trend towards increased recovery from tinnitus.

KEYWORDS:

Tinnitus, Auditory Nerve, Noise Induced Hearing Loss, Cochlea, Sarcosine, glycine, Piribedil, Cochlear Nucleus

List of abbreviations:

ABR - Auditory Brain Stem Response
ASR - acoustic startle reflex
BBN - Broad Band Noise
GI - Gap Inhibition
IHC - inner hair cells
OHC - outer hair cells
PPI - pre-pulse inhibition

II. BODY

GOALS from Statement of Work and Accomplishments towards Goals:

Aim One: MECHANISMS

1a: Determine if a small arms fire - like noise (50 biphasic impulses via a compression driver over a 2.5 minute period at a 152 dB SPL impulse level) will induce tinnitus in the rat model and, if so, what is the incidence of this noise-induced tinnitus in groups of rats exposed to the noise.

Timeline: Determine initially in Year One and then following (in control, non-treated animals) over the course of studies in following years.

Significant results / key outcomes: Development of tinnitus was identified based on a reduction in gap inhibition of the acoustic startle reflex (ASR) without reduction in pre-pulse inhibition (PPI) of the ASR (see next paragraph). If there was reduction in both gap inhibition and in PPI this was considered an auditory processing deficit that could include tinnitus, but could not be unambiguously identified as tinnitus. Year One studies showed that approximately 50% of the animals in a typical group of rats exposed to the small arms fire-developed reduced gap inhibition (e.g. Figure One), approximately one third of the noise exposed animals had reduced gap inhibition without reduced PPI and approximately 20% had both reduced gap inhibition and reduced PPI. This induction of tinnitus in approximately one third of the animals in a typical noise-exposed group was consistent over the next two years of studies in rats receiving this noise exposure and not in “sham” noise exposure animals.

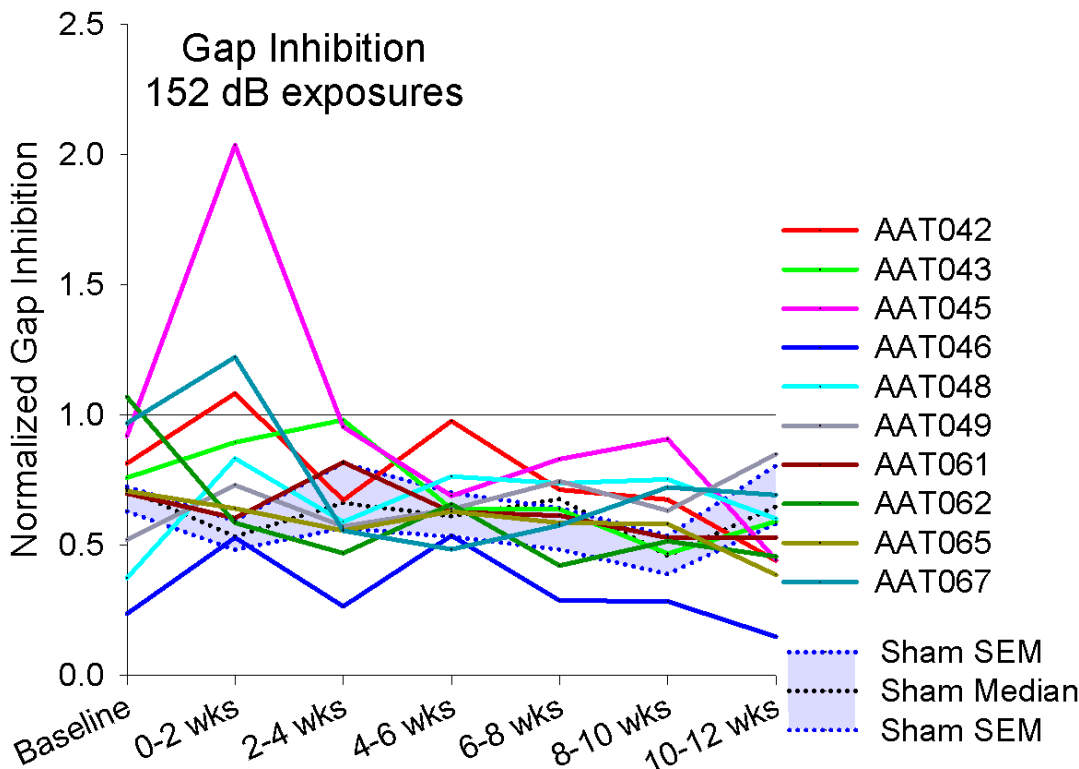


FIGURE ONE: Individual gap inhibition results from ten noise treated animals compared to the sham treated animals (gray shaded area). Approximately half of the noise exposed animals show reduced gap inhibition compared to the sham treated animals suggesting the presence of tinnitus.

HOW PRESENCE OF TINNITUS WAS DETERMINED: Data analysis methods were based on Li et al (2013). Each animal's data was divided into 2 week blocks (baseline, 0-2 weeks post exposure, 2-4 weeks post, and 4-6 weeks post, etc.). Animals were eliminated if their startle amplitude was too small to differentiate it from the “no startle” condition, ie “non-jumpers.” This was rare. For each 2 week block, a mean startle amplitude (no gaps, no prepulses) was calculated. Any jumps (for any condition) that exceed 2 standard deviations from this mean were considered outliers and not included in data analysis. For each condition

(Broad Band Noise (BBN), narrow band noises, various levels), an animal's data was only included (for that condition) if the baseline adjusted startle amplitude (eg: startle with gap / startle without gap) was less than 0.9. Each condition therefore had a different “n” of included animals.

Sham exposed animals were used as the standard to create the following three categories in the noise exposed animals:

- Animals with no changes to GI/PPI (compared to shams).
- Animals with changes only to GI (used to define presence of “tinnitus”).
- Animals with changes to both PPI and GI (auditory processing deficit that could include tinnitus).

Animals with changes only to PPI were rare (only two over the course of three years of noise exposures) and without explanation.

1b: Determine if the appearance of the noise-induced tinnitus correlates with the extent of loss of sensory cells (hair cells) – will there be greater loss of hair cells in the rats that develop tinnitus following noise versus those that get the same noise exposure but do not develop tinnitus

Timeline: The “in-life” noise exposures and assessment of Gap Inhibition to determine induction of tinnitus was carried out in Year One (see Aim 1a above). The post-life assessment of cochleae for hair cell loss was done in Year Two, and determination of relationship followed. Follow-up assessments in control, non-treated animals continued over the course of studies in following years.

Significant results / key outcomes: Hair Cell loss was determined using phalloidin stained surface preparations of noise exposed cochlea, generating a cytochleogram (Figure Two). Hair cell loss was compared in noise-exposed animals that developed tinnitus (based on loss of gap detection and no loss of PPI) compared to noise exposed animals without loss of gap detection. While there was a trend towards animals with more hair cell loss being more likely to have tinnitus, no significant relationship was found between the amount of hair cell loss and whether a noise exposed rat did or did not develop tinnitus following the noise.

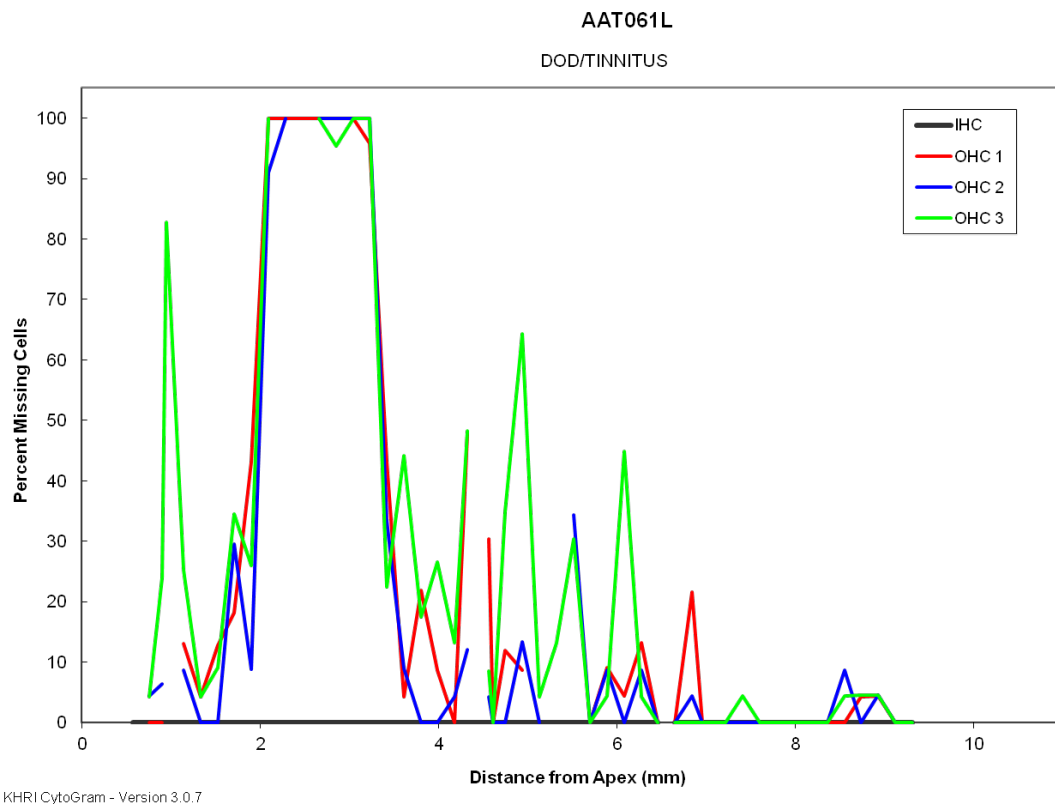


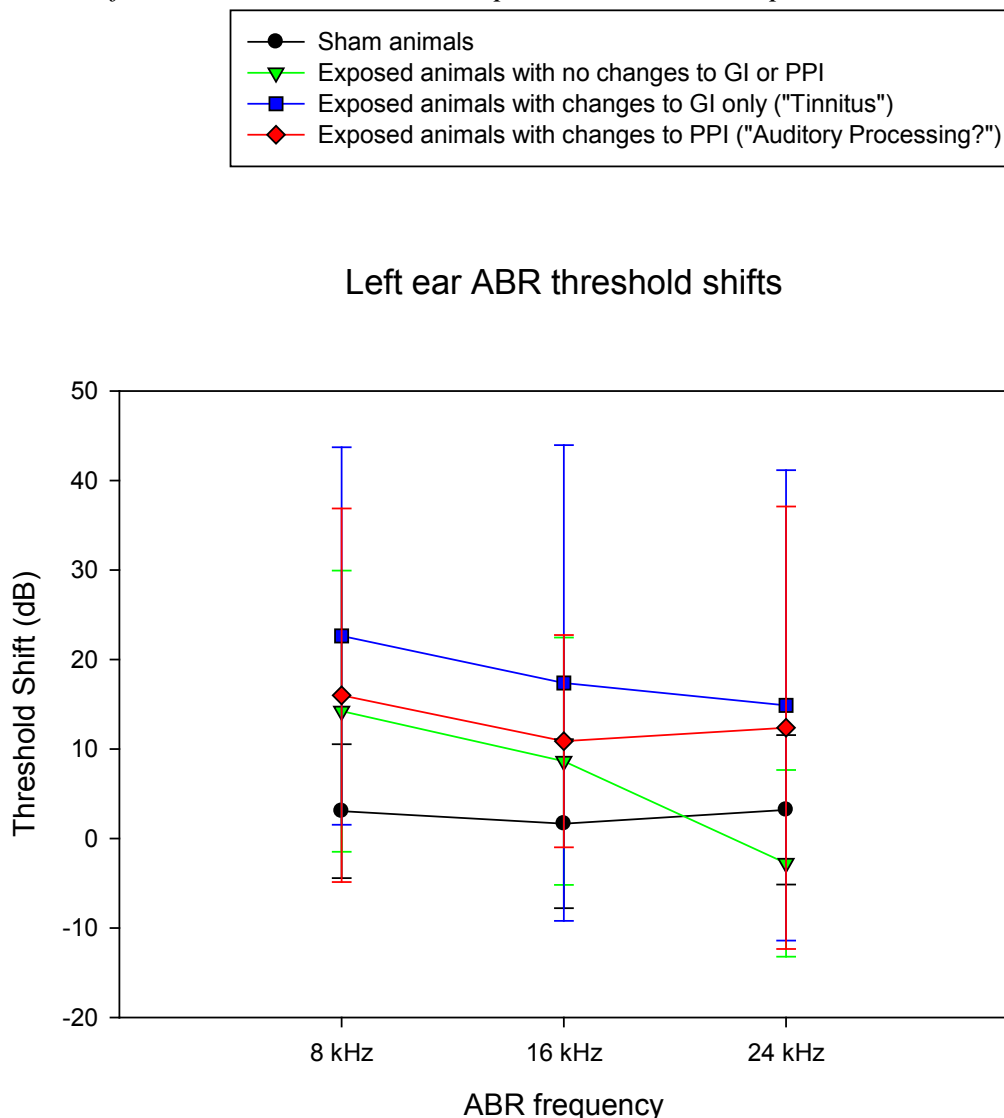
Figure Two: Cytochleogram plotting the percent of hair cell loss by position along the cochlear spiral with apex to left and base to the right. IHC = inner hair cells, OHC1,2,3 = outer hair cells in rows 1, 2 and 3 respectively. The plot above (AAT061) shows hair cell loss in a rat two months after the noise exposure. This rat received no preventive/protective treatments.

1c: Determine if the appearance of the noise-induced tinnitus correlates with the amount of hearing loss – will rats that develop tinnitus following noise have more hearing loss than those that get the same noise exposure but do not develop tinnitus.

Timeline: The “in-life” noise exposures and assessment of Gap Inhibition to determine induction of tinnitus was carried out in Year One (see 1a above). Final auditory brain stem response measure was made just before animals were terminated for post-life histological assessments, this continued into Year 2. Threshold shifts (comparison of hearing thresholds before noise and final hearing thresholds) were used as a measure of hearing loss.

Significant results / key outcomes: A comparison was made to determine if there was greater hearing loss (threshold shift in the Auditory Brain Stem Response - ABR) in noise exposed rats that developed tinnitus compared to noise exposed rat that did not develop tinnitus. As a group the animals developing tinnitus following noise exposure had larger threshold shifts than those that did not develop tinnitus, especially at 24 kHz (Figure 3), however, the differences were not significant because of large variability. When the relationship was examined on an individual rat basis no correlation was found.

Figure Three: Comparison of threshold shift of the auditory brain stem response (ABR) in rats receiving sham noise exposure (black circles), rats receiving noise exposure and not developing tinnitus (green triangles) and rats receiving noise exposure that did develop tinnitus (blue squares). There was no correlation between amount of threshold shift and whether or not noise exposed animals developed tinnitus.



1d: Determine if the appearance of the noise-induced tinnitus correlates with loss of Inner Hair Cell – Auditory Nerve Connections, will rats that develop noise-induced tinnitus have greater loss of Inner Hair Cell – Auditory Nerve Connections compared to those that do not develop tinnitus?

Timeline: The “in-life” noise exposures and assessment of Gap Inhibition to determine induction of tinnitus was carried out in Year One. The post-life assessment of cochleae for inner hair cell – auditory nerve connections was done in Year Two, and determination of correlation followed. Follow-up assessments in control, non-treated animals continued over the course of studies in following years.

Significant results / key outcomes: Loss of connections correlated with development of tinnitus. CTBP2 immunostaining of pre-synaptic ribbons was used as a marker for inner hair cell – auditory nerve synaptic connections and assessed in three regions of interest along the length of the cochlear spiral. The number of connections per inner hair cell was compared in noise exposed animals that developed tinnitus versus noise exposed animals that did not. There was a significant ($p=0.019$) decrease in the number of connections in animals that developed tinnitus (blue squares in Figure Four) compared to noise exposed animal that did not develop tinnitus (green triangles in Figure Four) in the most basal region of interest, consistent with results from Singer et al, (2013) which correlated loss of CTBP2 ribbons to incidence of tinnitus following a different noise exposure condition and with behavioral testing. There was also a significant decrease in connections in the animals with both reduced Gap Inhibition and reduced PPI.

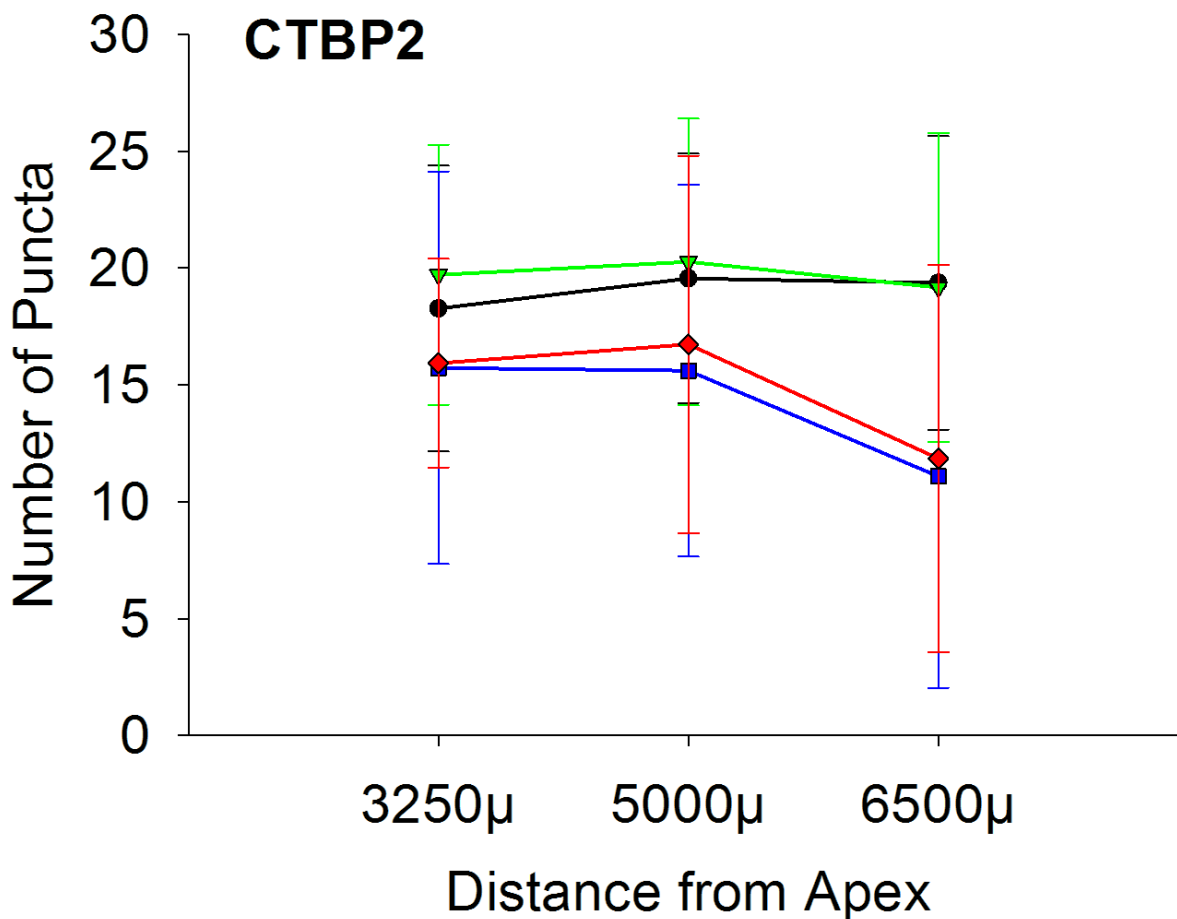


Figure Four: Comparison of the average number of CTBP2 puncta per inner hair cell (marker for the number of inner hair cell – auditory nerve terminals) in three regions of interest along the cochlear spiral. Compared among four groups of animals, rats receiving sham noise exposure (black circles), rats receiving noise exposure and not developing tinnitus (green triangles), rats receiving noise exposure that did develop tinnitus (blue squares) and rats receiving noise exposure that has changes to pre-pulse inhibition (PPI). Rats receiving noise but not developing tinnitus (green triangles) had numbers that were comparable to rats receiving no noise / shams (black circles). On the other, there were large decreases in the number of connections in the noise exposed rats that did develop tinnitus (blue squares).

Our studies were designed based on literature that showed noise-induced loss of connections with the percent loss maintained over the course of time (Kujawa and Liberman, 2006, 2009). More recent studies have shown that reconnection and reinnervation following noise can occur over the course of several weeks (Shi et al, 2012, Liu et al, 2013) with variable efficiency. This then adds another variable, the amount of re-innervation / reconnection that could be occurring in each cochlea. This could explain why we found less loss in the more apical regions of interest and may be why results did not reach significance in all regions of interest assessed. We have therefore begun new independent studies to compare the number of connections 2-3 days following the noise exposure to the number 4-6 weeks later. Greater loss at 2-3 days than at 4-6 weeks would indicate reconnection. We are also using GAP-43 (a growth cone associated protein) as a marker for regrowth. In studies (with a different noise) we find GAP-43 immuno-labeling under hair cells, evidence of regrowth following noise which could also apply to the small arms fire noise exposure condition.

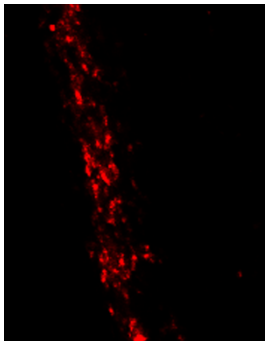


Figure Five – A surface preparation of the cochlear spiral from a noise exposed rat assessed 4 weeks following the noise. Terminals at the bases of Inner Hair Cells are immune-labeled with GAP-43 a marker for regrowth of connections.

1e: Determine if the appearance of the noise-induced tinnitus correlates with the amount of loss of Auditory Nerve Connections in the cochlear nucleus – will rats that develop tinnitus following noise versus have greater loss of Auditory Nerve Terminals compared to those that get the same noise exposure but do not develop tinnitus.

Timeline: Initial studies in Years Two - Three, with assessment and correlation in Year Three - Four and then follow-up (in control, non-treated animals) over the course of studies in following years.

Significant results / key outcomes: These studies are **incomplete**. These assessments are still underway.

AIM TWO: MECHANISM – BASED INTERVENTIONS TARGETING INNER HAIR CELL – AUDITORY NERVE CONNECTIONS

GOALS: Determine if an anti-apoptotic intervention (ACEMg) and/or an anti-excitotoxic intervention (Memantine & Piribedil) will reduce loss of IHC-AN connections and prevent or reduce the appearance of noise-induced tinnitus.

2A: Determine if an anti-apoptotic intervention (ACEMg) and/or an anti-excitotoxic intervention (Memantine & Piribedil) will reduce the noise-induced loss of IHC-AN connections.

Timeline & Milestones: The studies in Aims 2a were performed in years 1 & 2, assessment in years 2 - 3.

Significant results / key outcomes: Results showed that treatments provided significant protection from noise-induced loss of IHC-AN connections. When treated with a combination therapy of ACEMg, Piribedil and Memantine there was significantly less loss of IHC-AN connections from the small arms fire-like noise exposure in the most basal region of interest. There was a significantly greater number of connections compared to animals that got the same noise but no treatments (Figure Six). Treatment with only Ace-Mg (Vitamins A, C and E plus magnesium) was ineffective, with the same noise-induced loss of connections as in animals with noise and no treatments. Treatment with just Piribedil and Memantine reduced the noise-induced loss of connections so that the loss was no longer significant, however, when compared to the amount of loss without treatment it did not reach significance. So the conclusion is that the combination of treatments is synergistic and the most effective, with greatest contribution from Memantine and Piribedil. These results will be presented as an abstract at the 2015 meeting of the Association for Research in Otolaryngology and is part of the manuscript in preparation.

It is interesting that this protection was not observed in the more apical regions of interest, perhaps because there was considerably less loss of connections induced by the noise and thus less room for protection. This may have been influenced by reconnection / regrowth as an additional variable. This is discussed later.

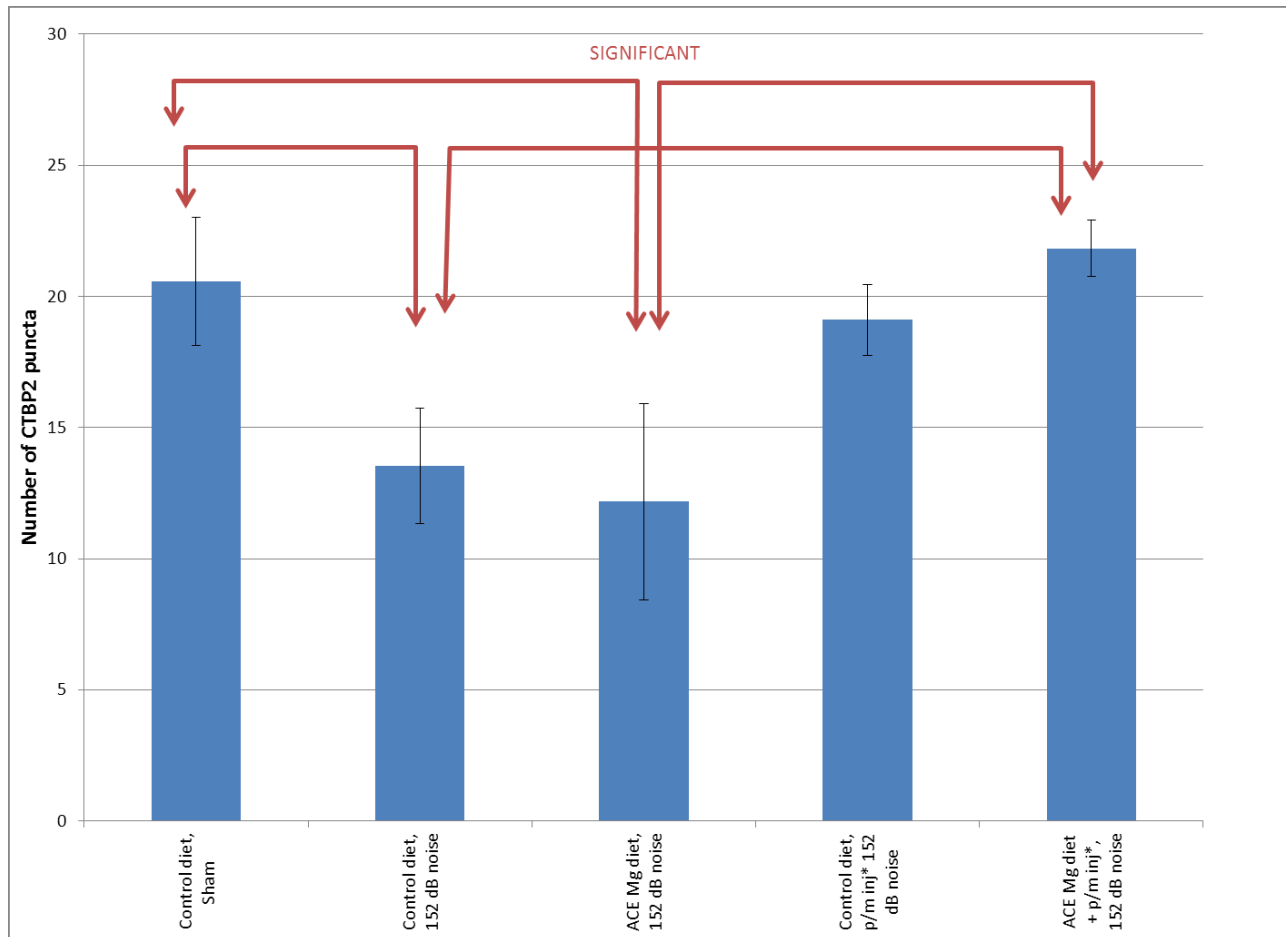


Figure Six: Treatment with anti-excitotoxic agents plus anti-oxidants prevents noise induced loss of Inner Hair Cell –Auditory Nerve connections. This bar graph compares the average number of CTBP2 puncta (marker for inner hair cell –auditory nerve connections) per inner hair cell in the basal turn of the cochlear spiral in five conditions: Controls (received only a Sham Noise exposure and no treatments); Animals getting a noise exposure and no protective treatments; Animals getting noise exposure and only ACE-Mg protective treatment; Animals getting noise exposure and only memantine and piribedil protective treatments; Animals getting noise exposure and ACE-Mg, memantine and piribedil protective treatments. There is no significant loss of connections (compared to control no-noise shams) in the noise exposed animals getting either memantine and piribedil only or memantine, piribedil and ACE-Mg protective treatments. Noise exposed animals without any protective treatments or with only ACE-Mg protective treatments both had significant loss of connections. The memantine, piribedil and ACE-Mg protective treatment group had a significantly greater number of connections than the noise-exposed group without protective treatment.

2B: Determine if an anti-excitotoxic intervention (Memantine & Piribedil) and/or an anti-apoptotic intervention (ACEMg) will reduce the incidence of noise-induced tinnitus in treated animals compared to untreated ones.

Timeline & Milestones: The studies in Aims 2b were performed in years 1 and 2, with assessment in years 2-3.

Significant results / key outcomes: The incidence of tinnitus (the percent developing reduced gap detection without reduced PPI following the noise exposure or sham) is shown as the black bars in Figure Seven.

Combined treatment with anti-excitotoxicity agents (Piribedil and Memantine) plus anti-oxidants (Vitamins A, C and E plus magnesium) resulted in a significantly decreased incidence of noise-induced tinnitus (12.5% developing tinnitus) compared to animals receiving just the noise exposure and no preventive treatment (29% developing tinnitus) (Figure Seven). When animals showing both reduced Gap

Inhibition and reduced PPI following noise (or sham) are added (grey bars) then only the combined anti-excitotoxicity and anti-oxidant treatment or anti-excitotoxicity treatments reduced the incidence, and ACEMg (anti-oxidants) alone was not effective.

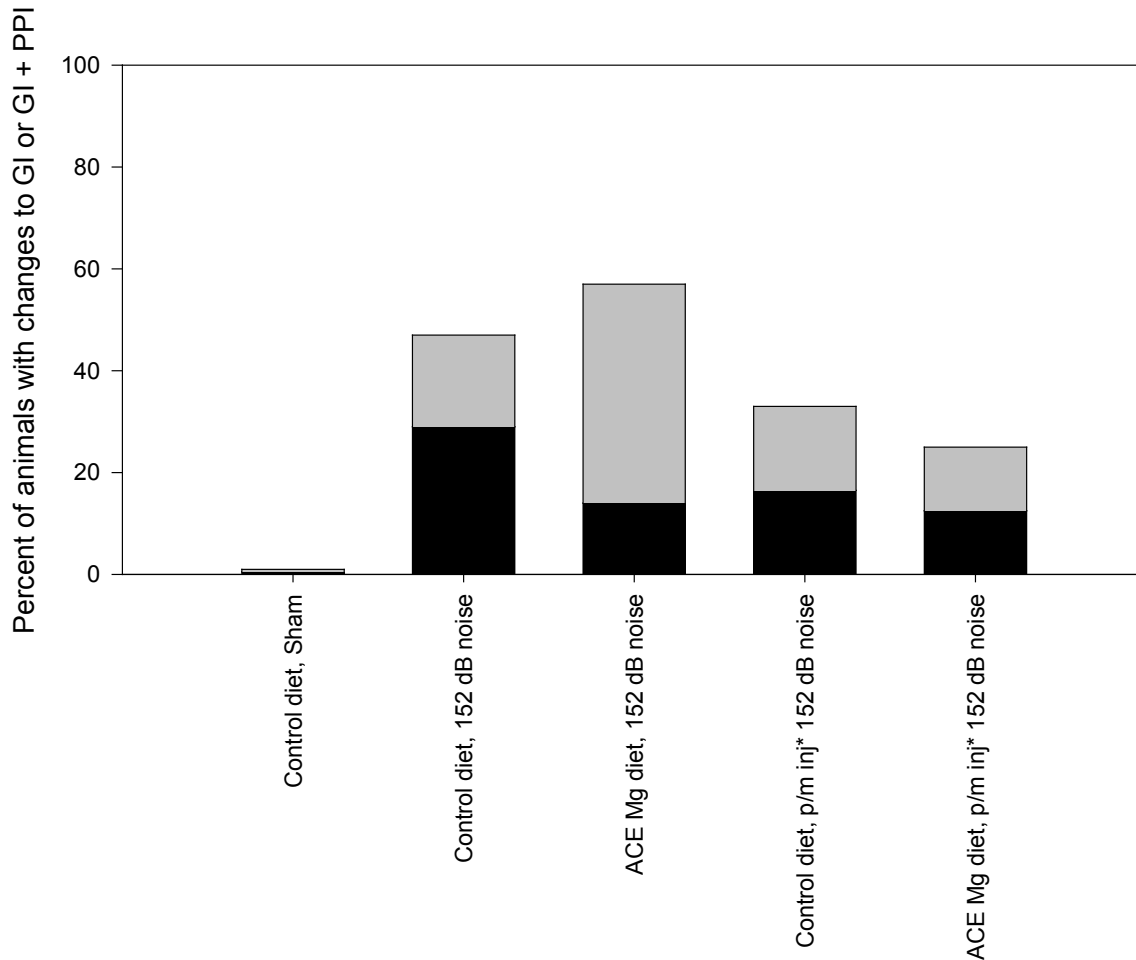


Figure Seven: Treatment with anti-excitotoxic agents and/or anti-oxidants reduced the incidence of noise induced tinnitus. The black bars show the percent of animals with reduced gap inhibition without reduced PPI (our criteria for development of tinnitus). Grey bars add the percent of animals showing both reduced Gap Inhibition and reduced PPI. 29% of the animals receiving the small arms fire – like noise and no protective treatment (labeled as Control Diet) developed tinnitus. All three types of tested treatments reduced the incidence of tinnitus, reduced to 14% in ACEMg only group, to 13% in the combined ACEMg plus Memantine and Piribedil treatment group, and to 17% in Memantine and Piribedil only treatment group. When animals that also had reduced PPI are added to generate the total percent with reduced Gap Inhibition (both with and without reduced PPI) then only the combined ACEMg plus Memantine and Piribedil treatment group and the Memantine and Piribedil only treatment group showed reduced incidence.

It is interesting to compare results in Figures Six and Seven and the influence of reducing loss of Inner Hair Cell – Auditory Nerve connections. Only the combined anti-excitotoxicity and anti-oxidant treatment or anti-excitotoxicity treatment alone reduced loss of connections and only these treatments reduced the percent of animals showing reduced Gap Inhibition (without regard to PPI). This would suggest a relationship between loss of connections and reduced Gap Inhibition. When the additional qualifier for our identification of tinnitus was added (reduced Gap Inhibition without reduced PPI) then all treatments, including anti-oxidants, were effective in reducing incidence. Anti-oxidants may contribute towards reducing development of tinnitus, but perhaps not through the mechanism of reducing loss of connections. Loss of connections could be involved both in generation of tinnitus and in generation of auditory processing disorders.

Unanticipated Results and Problems: As mentioned previously our studies were designed based on literature that showed noise-induced loss of connections with the percent loss maintained over the course of time (Kujawa and Liberman, 2006, 2009) and more recent studies have shown that reconnection and reinnervation following noise can occur over the course of several weeks (Shi et al, 2012, Liu et al, 2013) with variable efficiency. This then adds another variable, the amount of re-innervation / reconnection occurring in each cochlea. Protection could have been influenced by subsequent regrowth / reconnection in non-treated cochlea in some regions of interest (more apical ones where reconnection seems to be more efficient). It also raises the question of how soon the reconnection occurs after the noise exposure and if a period of time before reconnection is sufficient to induce tinnitus and if so, how long a period of time. Future studies will be needed to address this question.

AIM THREE: MECHANISMS IN THE AUDITORY BRAIN STEM

Aim 3a - Mechanisms: Test the hypothesis that loss of Auditory Nerve terminals (marked by VGLUT1 immunolabel) on neurons in the VCN and DCN (Aim 1b) will be followed by an increases in inappropriate excitatory influences (VGLUT2 marking non-auditory nerve glutamatergic terminals and VAT marking cholinergic terminals) and decreases in inhibitory amino acid terminals (marked by VGAT), changing the balance in excitatory – inhibitory influence towards reduced inhibitory synaptic strength.

Timeline & Milestones: The studies in Aims 1 were performed in years 1 – 3. Animals have been terminated and brain stems prepared for cryostat sectioning. While assessments had been planned for years 2-3, these are incomplete and still underway.

Significant results / key outcomes: INCOMPLETE: Assessment is still underway.

Aim 3b - Mechanisms: Test the hypothesis that decreases in genes and proteins associated with inhibitory synaptic strength and changes in ion channels that regulate neuronal excitability towards increased excitability will be found in the cochlear nucleus and inferior colliculus of animals with noise-induced chronic tinnitus and not in animals receiving comparable noise exposure but not developing tinnitus.

Timeline & Milestones: The collection of RNA from cochlear nucleus and inferior colliculus was completed in years 2-3. The qRT-PCR for different gene candidates is still underway.

Significant results / key outcomes: INCOMPLETE: Initial results indicate several of candidates with noise-induced changes in gene expression, with fewer showing differences in animals showing tinnitus versus those that did not develop tinnitus. An L-type calcium channel did show differences between animals developing tinnitus and those that did not (Figure Eight). Additional candidates are currently being assessed.

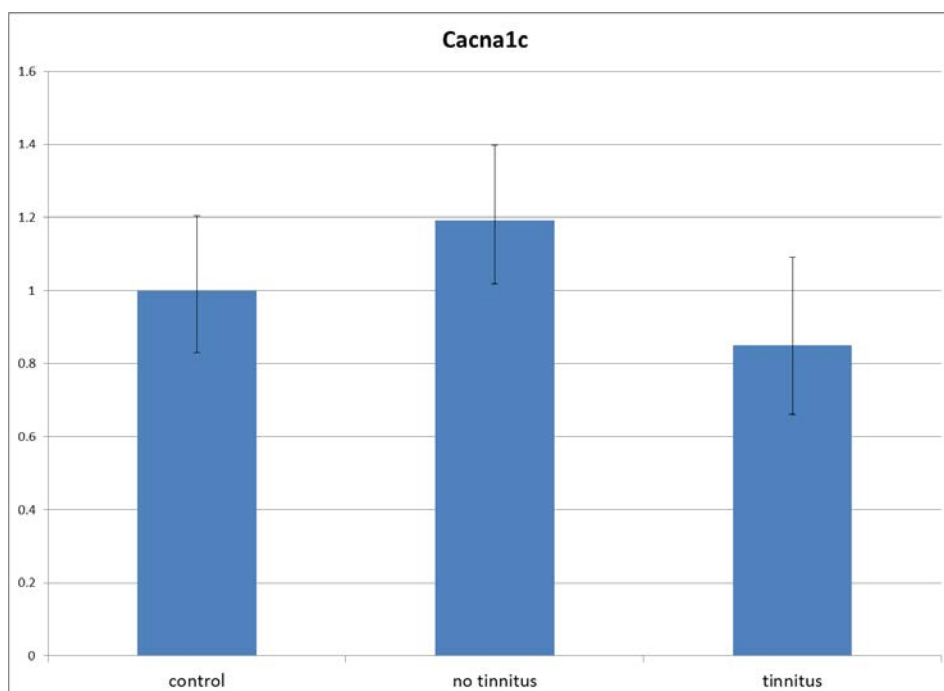


Figure Eight – Expression of *Cacna1c* (Calcium channel, voltage-dependent, L type, alpha 1C subunit) in the Inferior Colliculus compared between control rats (sham noise exposure), animals receiving noise exposure that developed tinnitus (no tinnitus) and those that develop tinnitus following the noise (tinnitus). Expression increased in animals that did not develop tinnitus and decreased in those that did. The difference in expression between no tinnitus and tinnitus was significant ($p = 0.03$)

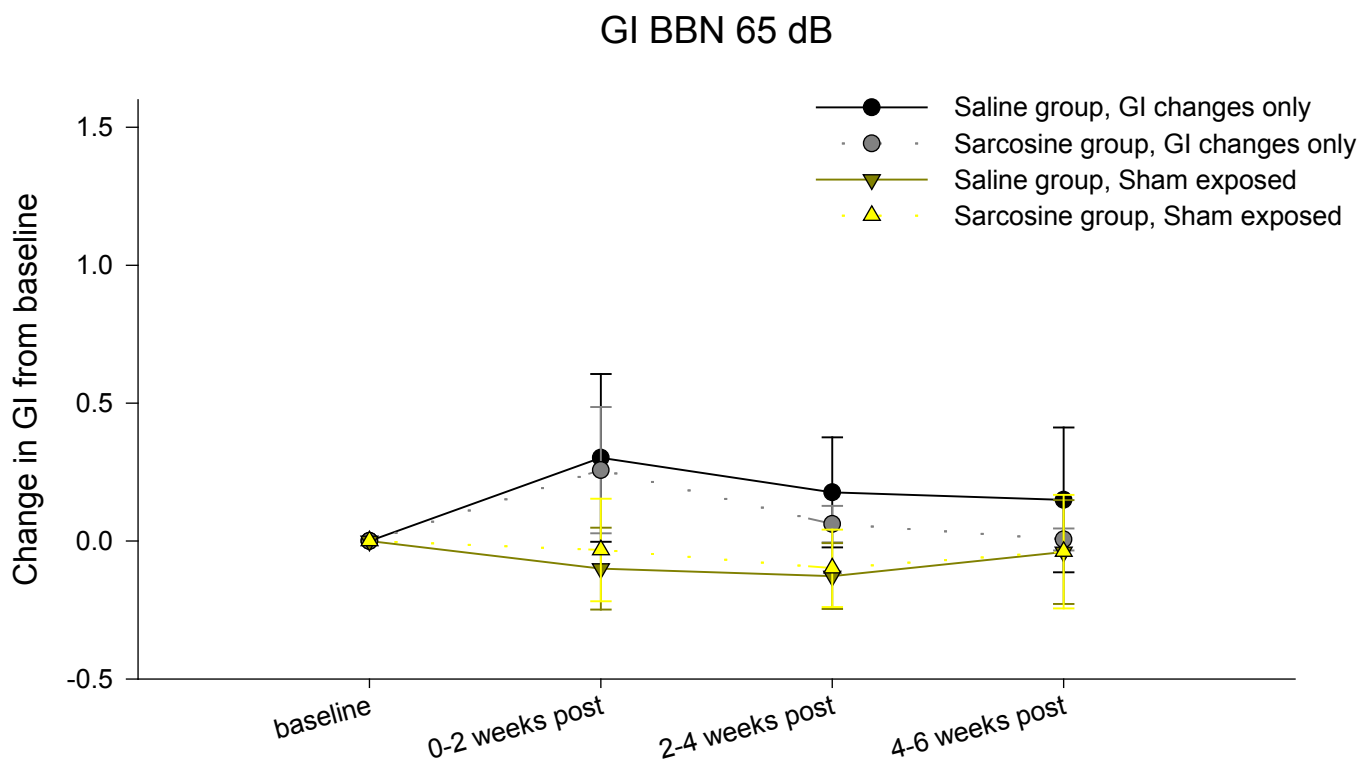
AIM FOUR: MECHANISM - BASED INTERVENTIONS TARGETING THE AUDITORY BRAN STEM

Aim 4 - Test the hypothesis that treatments which reverse the increased excitability will reduce or eliminate noise-induced chronic tinnitus. Therapy is with SARCOSINE, which increases inhibitory synaptic strength by decreasing uptake of glycine or Retigabine which stabilizes membrane potential and neuronal excitability by opening Kv7.2-7.5 (KCNQ2-5) channels.

Timeline & Milestones: The Sarcosine studies in Aim 4 were performed in year 3. Assessments were completed at the end of year 3. Retigabine assessment, comparable to what was proposed here, appeared in the literature from a different group (Li et al, 2013) and therefore became lower priority and incomplete.

Significant results / key outcomes: Sarcosine reduces the rate of take up of glycine from synapses and so increases the inhibitory influence. Studies tested if sarcosine given before or after a small arms fire-like noise exposure would reduce the induction of tinnitus. Small arms fire-like noise exposed animals receiving saline (controls) or sarcosine (treatment group) both showed comparable changes in Gap Inhibition at 0-2 weeks with a significant change from baseline as a group, they began to diverge at 2-4 weeks and by 4-6 weeks the sarcosine treated animals were more comparable to the sham (no noise) animals than the noise rats exposed that got control (saline) treatment (Figure Nine). The differences, however, did not reach significance, so were only a strong trend. At 1-2 weeks the incidence of tinnitus (number of animals meeting our criteria for tinnitus divided by the total number of animals in the group) was identical for the sarcosine treated and non-treated noise exposed animals. At 2-4 weeks and 4-6 weeks the incidence of tinnitus in the sarcosine treated noise exposed group was 50% of the incidence in the untreated noise exposed group, again supporting sarcosine increasing recovery.

FIGURE NINE – Changes in Gap Inhibition over six week weeks following noise exposure in four groups, two with Sham Noise (one with sarcosine given over the six week period, one with just saline) and two with the small arms fire-like noise (one with sarcosine given over the six week period, one with just saline). Sham groups did not show changes. There was comparable decline in Gap Detection in both noise exposed groups during the first two week, some divergence during the next two weeks and during weeks 4-6 the noise exposed group with sarcosine treatment was comparable to the shams, while the noise exposed without treatment remained reduced.



Unanticipated Results and Problems:

As with Aims One and Two, our power analysis had been based on the expectation that noise exposed animals would fall either into a group with tinnitus (based on showing reduced gap detection) or without tinnitus (based on no significant changes in gap detection). However, a third group emerged, animals with reduced gap detection and reduced pre-pulse inhibition. Pre-pulse inhibition should not be effected by tinnitus. When an animal presents with both PPI and reduced Gap Detection, this can raise questions as to whether reduced Gap Detection is from tinnitus or from another central auditory processing disorder. We therefore did not include these animals in the “tinnitus” group, but considered them separately. This reduced our “n” and reduced the power and could explain why difference (Figure Nine) did not reach significance. Future studies will therefore be needed to appropriately test if sarcosine can improve recovery from tinnitus.

4. SUMMARY OF KEY RESEARCH ACCOMPLISHMENTS:

- **A small arms fire-like noise exposure (50 biphasic impulses via a compression driver over a 2.5 minute period at 152 dB SPL impulse level) will induce tinnitus (based on reduction in gap inhibition without reduction in pre-pulse inhibition) in approximately one third of the rats receiving this noise. An additional 20% of the rats have losses in both gap detection and pre-pulse inhibition.**
- **This small arms fire-like noise results in a significant loss of Inner Hair Cell – Auditory Nerve Connections.**
- **Treatment with anti-excitotoxicity agents (Memantine and Piribedil) plus anti-oxidants (vitamins A, C and E), magnesium (ACE-Mg) prevents this noise-induced loss of Inner Hair Cell – Auditory Nerve connections.**
- **This treatment also decreases the incidence of tinnitus. Only 13% of animals receiving this treatment developed noise-induced tinnitus compared to 29% of animals in the group receiving the noise and no treatments.**
- **Sarcosine treatment to enhance glycine inhibition in the central auditory pathways does not provide a significant decrease in the incidence of tinnitus when provided either before or immediately after the noise exposure. However, when given over a period of weeks following the noise it may improve recovery from tinnitus.**

5. CONCLUSIONS & IMPACT

The important novel finding is that treatment with anti-excitotoxicity agents (Piribedil and Memantine) given prior to the small arms fire-like noise provides protection from the noise-induced loss of Inner Hair Cell synapses in the rat model and reduces the incidence of tinnitus. Piribedil and memantine are already clinically approved and applied for other disorders and could be repurposed to prevent noise-induced loss of Inner Hair Cell – Auditory Nerve connections and prevent tinnitus. The loss of connections has also been shown to reduce dynamic range which is believed to degrade speech processing in a noisy environment. Our studies suggest it could also contribute to other auditory processing disorders. Protection from development of tinnitus and reducing auditory processing disorders would therefore have direct military relevance to preventing breakdown in communication in the battlefield, towards redeployment and towards later quality of life. Our studies also suggest that anti-oxidants may also contribute towards reducing development of tinnitus, but perhaps not through the mechanism of reducing loss of connections.

The next set of studies targeted the auditory brain stem and treatment (rather than prevention). It tested the effects of sarcosine, which increases inhibitory influence by decreasing uptake of glycine from synapses. The studies found that sarcosine when provided either shortly after the noise exposure or shortly before did not influence the induction of tinnitus. Interestingly, however, sarcosine when provided over a 6 week period following the noise showed a trend towards recovery in animals that developed tinnitus. Sarcosine is also already clinically approved and applied for other disorders. Future studies will be needed to follow up on recovery from tinnitus with more animals and more power.

Future plans (to accomplish the goals and objectives):

Several of the “incomplete” analyses are still underway, these are the assessment of transmitter terminals in the cochlear nucleus and gene expression assessments in the cochlear nucleus and inferior colliculus which will then be examined to determine if there is a correlation with tinnitus.

Further studies are necessary where “weak” correlation or not reaching significance (but close) was observed. Specifically will treatment with sarcosine over a course of weeks following development of tinnitus result in recovery and elimination of tinnitus.

Further studies will also be necessary to examine if there is “natural” reinnervation or reconnection following noise-induced loss of connections. This could explain the small, non-significant loss of connections in several regions of the cochlear spiral. Specific therapeutics to promote and increase reconnection could be an additional treatment option that should be tested in the future.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

ABSTRACTS

Karin Halsey; David Dolan; Josef Miller; Susan Shore; Jennifer Eberle; Ariane Kanicki; Susan DeRemer; Diane Prieskorn; Richard Altschuler. (2014) Impulse Noise Effects on ABR, Pre-Pulse Inhibition, Gap Detection, and Auditory Nerve Connections, Abstracts, Association for Research in Otolaryngology, 2014

Dave Dolan, Susan Shore, Josef Miller, Karin Halsey, Diane Prieskorn, Ariane Kanicki, Peter Ghisleni, Rick Altschuler. (2013) Simulated Small Arms Fire Exposure in Rats Results in Reduced Gap Inhibition of the Acoustic Startle Reflex Suggesting Induction of Tinnitus, Abstracts, Association for Research in Otolaryngology, 2013.

David Dolan, Karin Halsey, Peter Ghisleni, Richard Altschuler. (2012) Effects of Background Noise on the Acoustic Startle Response, Abstracts, Association for Research in Otolaryngology, 2012.

• **INVENTIONS, PATENTS AND LICENSES:** List all patents and licenses applied for and/or issued. Each entry must include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

"Nothing to report."

• **REPORTABLE OUTCOMES:** Provide a list of reportable products that have resulted from this research. Products are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and / or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes or similar products that may be commercialized.

"Nothing to report."

• **OTHER ACHIEVEMENTS:**

This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

"Nothing to report."

- **REFERENCES:**

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- **LIST OF PERSONNEL ON THIS AWARD:**

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Aaron Hughes
Capreece Holland
Lily Walker

A. SUPPORTING DATA:

FIGURE ONE: Individual gap inhibition results from ten noise treated animals compared to the sham treated animals (gray shaded area). Approximately half of the noise exposed animals show reduced gap inhibition compared to the sham treated animals suggesting the presence of tinnitus.

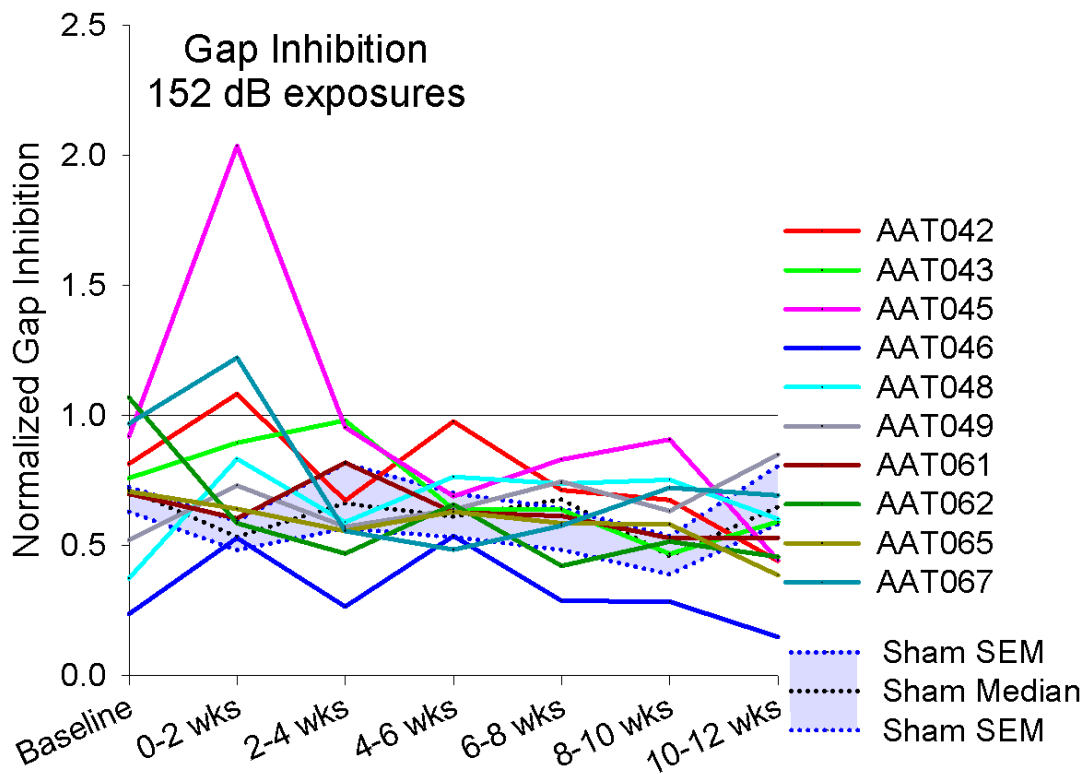
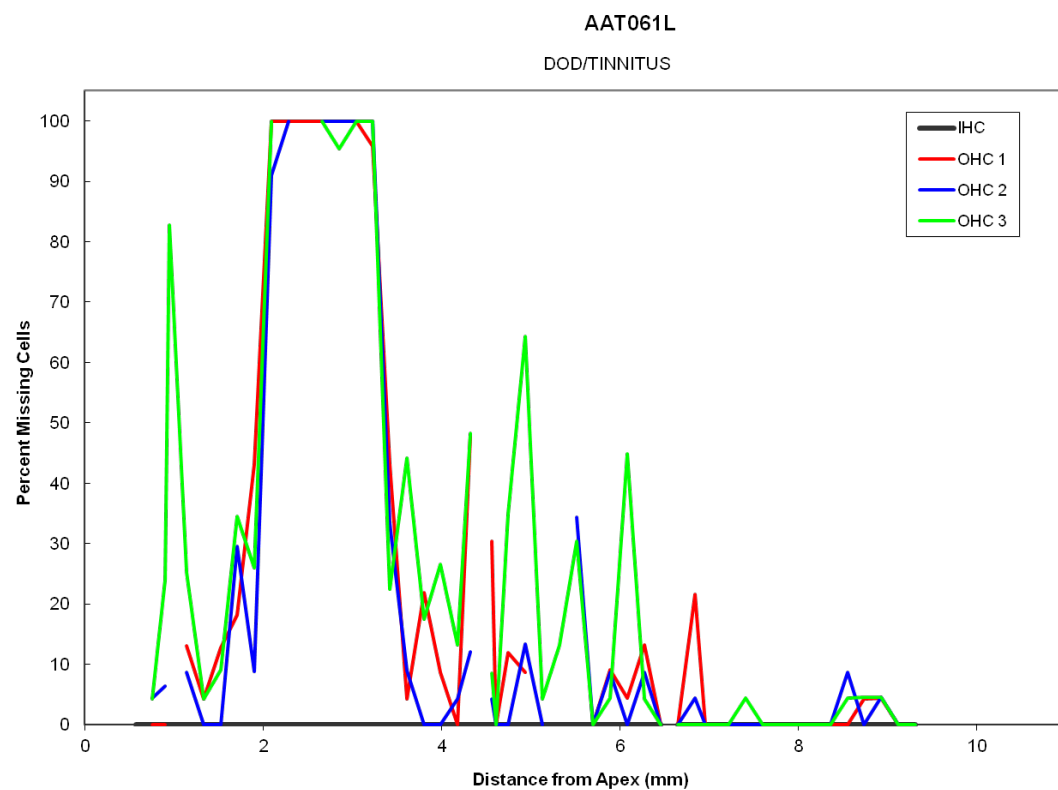


Figure Two: Cytocochleogram plotting the percent of hair cell loss by position along the cochlear spiral with apex to left and base to the right. IHC= inner hair cells, OHC1,2,3 = outer hair cells in rows 1, 2 and 3 respectively. The plot (AAT061) shows hair cell loss in a rat two months after the noise exposure. This rat received no preventive/protective treatments.



KHRI CytoGram - Version 3.0.7

Figure Three: Comparison of threshold shift of the auditory brain stem response (ABR) in rats receiving sham noise exposure (black circles), rats receiving noise exposure and not developing tinnitus (green triangles) and rats receiving noise exposure that did develop tinnitus (blue squares). There was no correlation between amount of threshold shift and whether or not noise exposed animals developed tinnitus.

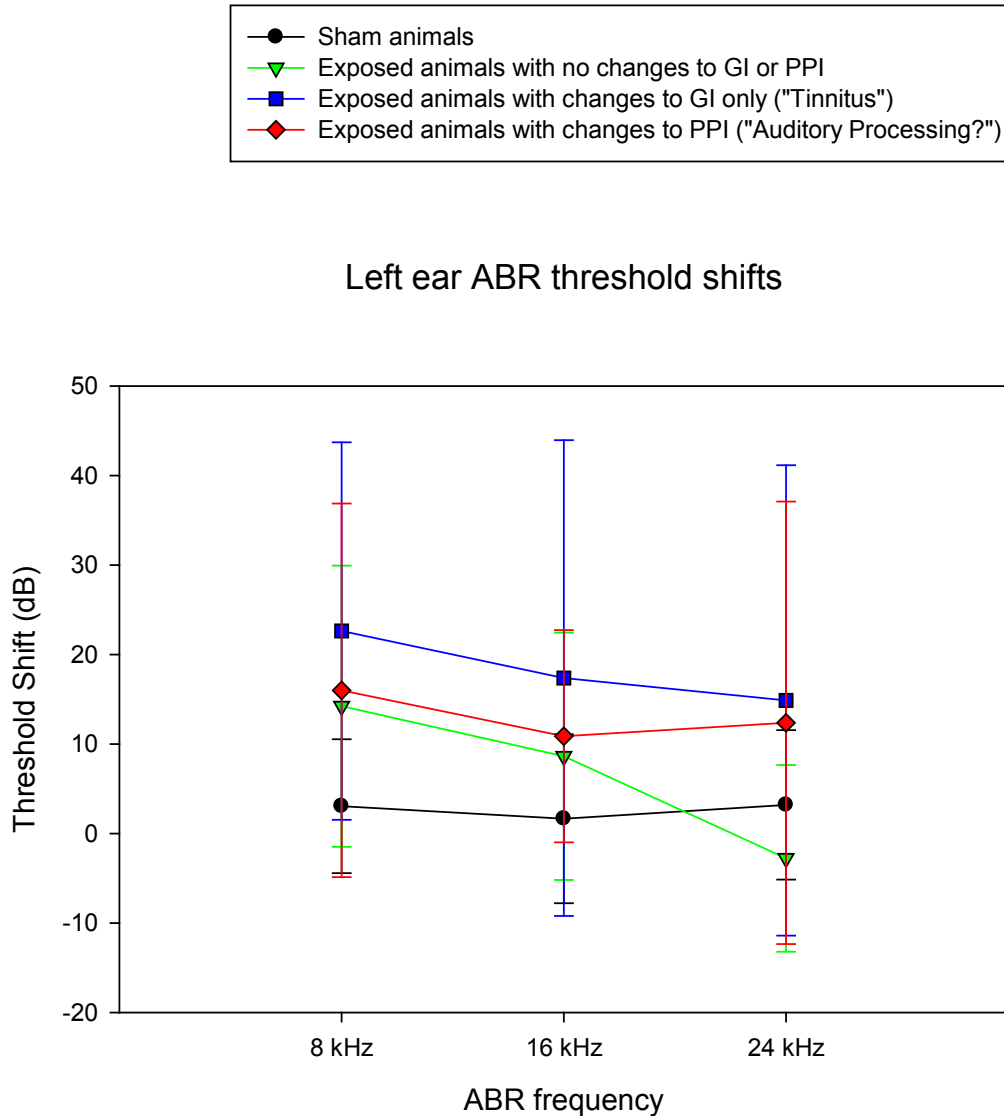


Figure Four: Comparison of the average number of CTBP2 puncta per inner hair cell (marker for the number of inner hair cell –auditory nerve terminals) in three regions of interest along the cochlear spiral. Compared among four groups of animals, rats receiving sham noise exposure (black circles), rats receiving noise exposure and not developing tinnitus (green triangles), rats receiving noise exposure that did develop tinnitus (blue squares) and rats receiving noise exposure that has changes to pre-pulse inhibition (PPI). Rats receiving noise but not developing tinnitus (green triangles) had numbers that were comparable to rats receiving no noise / shams (black circles). On the other, there were large decreases in the number of connections in the noise exposed rats that did develop tinnitus (blue squares).

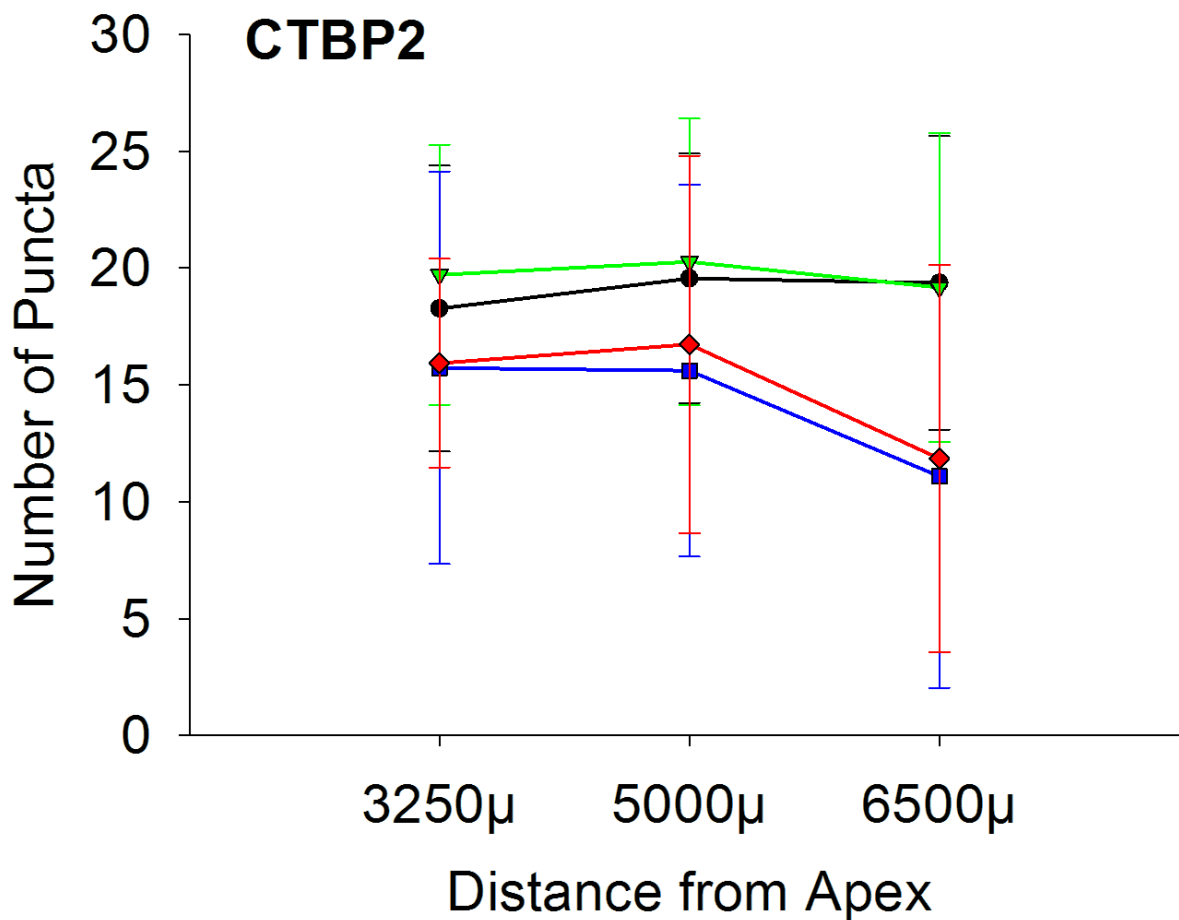


Figure Five – *A surface preparation of the cochlear spiral from a noise exposed rat assessed 4 weeks following the noise. Terminals at the bases of Inner Hair Cells are immune-labeled with GAP-43 a marker for regrowth of connections.*

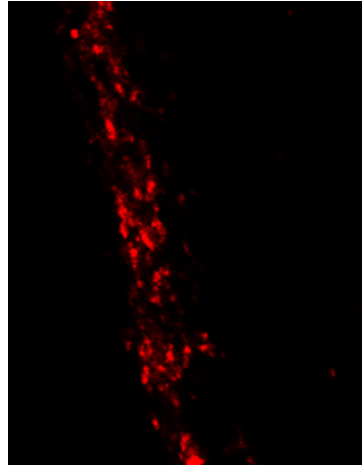


Figure Six: Treatment with anti-excitotoxic agents plus anti-oxidants prevents noise induced loss of Inner Hair Cell –Auditory Nerve connections. This bar graph compares the average number of CTBP2 puncta (marker for inner hair cell –auditory nerve connections) per inner hair cell in the basal turn of the cochlear spiral in five conditions: Controls (received only a Sham Noise exposure and no treatments); Animals getting a noise exposure and no protective treatments; Animals getting noise exposure and only ACE-Mg protective treatment; Animals getting noise exposure and only memantine and piribedil protective treatments; Animals getting noise exposure and ACE-Mg, memantine and piribedil protective treatments. There is no significant loss of connections (compared to control no-noise shams) in the noise exposed animals getting either memantine and piribedil only or memantine, piribedil and ACE-Mg protective treatments. Noise exposed animals without any protective treatments or with only ACE-Mg protective treatments both had significant loss of connections. The memantine, piribedil and ACE-Mg protective treatment group had a significantly greater number of connections than the noise-exposed group without protective treatment.

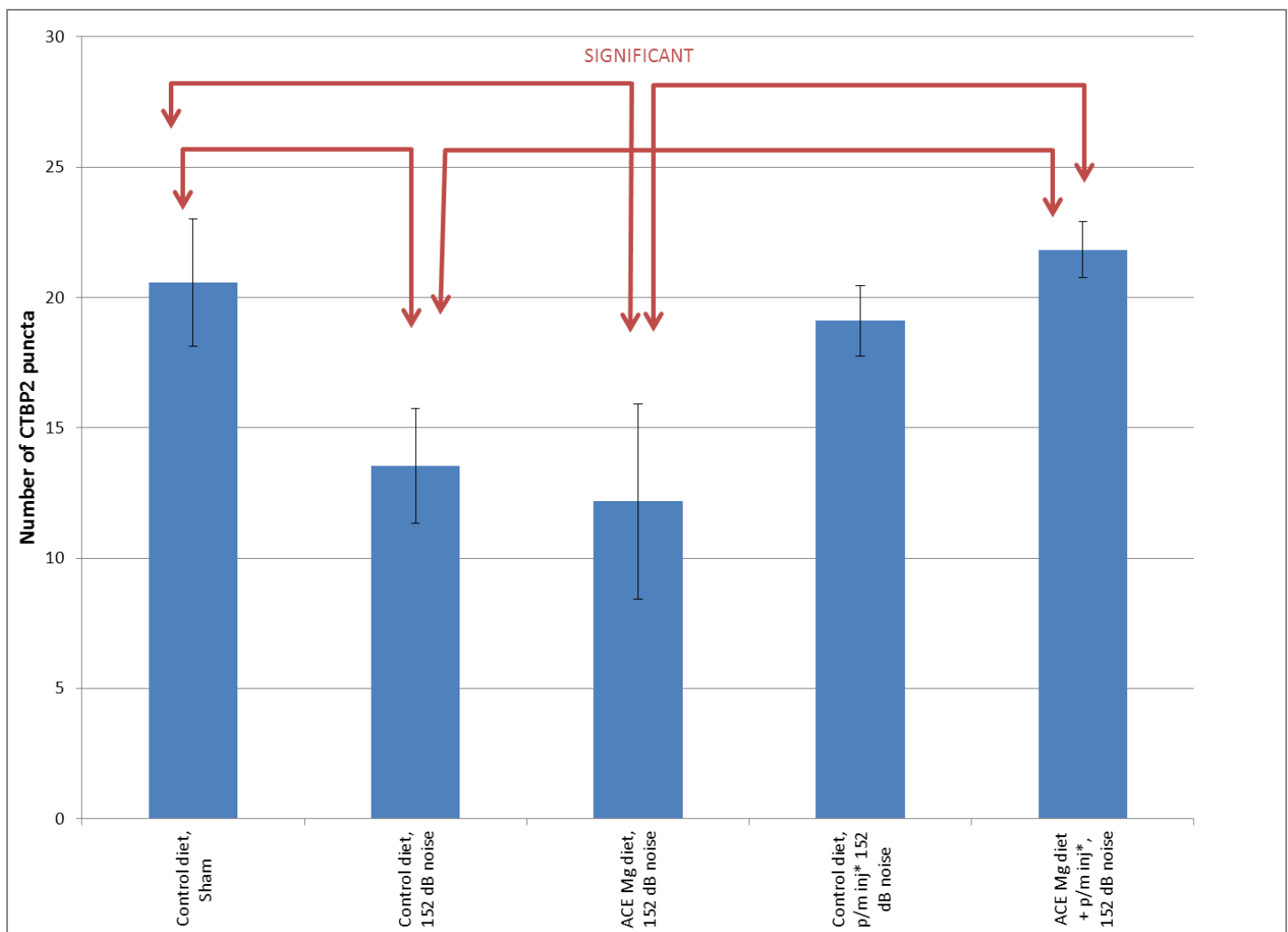


Figure Seven: Treatment with anti-excitotoxic agents and/or anti-oxidants reduced the incidence of noise induced tinnitus. The black bars show the percent of animals with reduced gap inhibition without reduced PPI (our criteria for development of tinnitus). Grey bars add the percent of animals showing both reduced Gap Inhibition and reduced PPI. 29% of the animals receiving the small arms fire – like noise and no protective treatment (labeled as Control Diet) developed tinnitus. All three types of tested treatments reduced the incidence of tinnitus, reduced to 18% in ACEMg only group, to 18% in the combined ACEMg plus Memantine and Piribedil treatment group, and to 19% in Memantine and Piribedil only treatment group. When animals that also had reduced PPI are added to generate the total percent with reduced Gap Inhibition (both with and without reduced PPI) then only the combined ACEMg plus Memantine and Piribedil treatment group and the Memantine and Piribedil only treatment group showed reduced incidence.

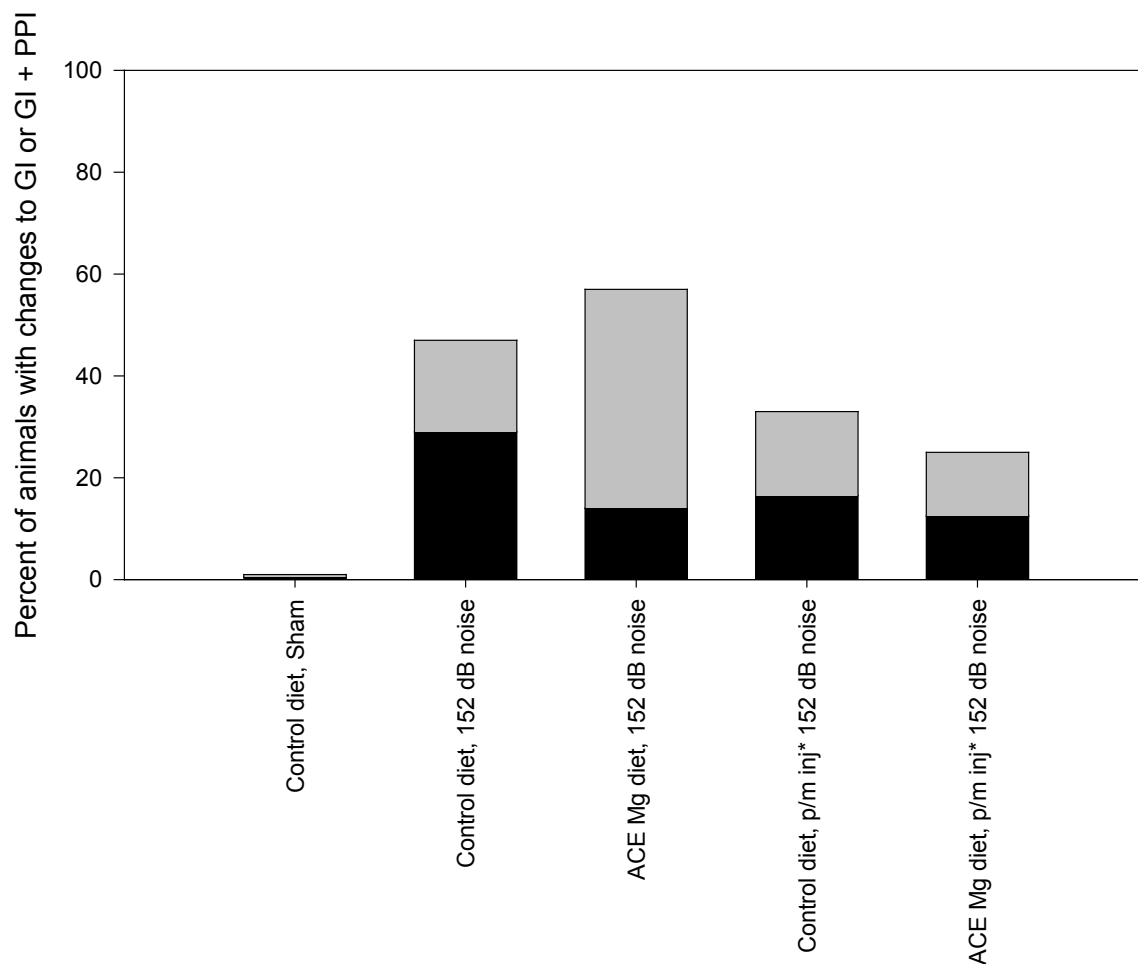


Figure Eight – Expression of *Cacna1c* (Calcium channel, voltage-dependent, L type, alpha 1C subunit) in the Inferior Colliculus compared between control rats (sham noise exposure), animals receiving noise exposure that developed tinnitus (no tinnitus) and those that develop tinnitus following the noise (tinnitus). Expression increased in animals that did not develop tinnitus and decreased in those that did. The difference in expression between no tinnitus and tinnitus was significant ($p = .03$)

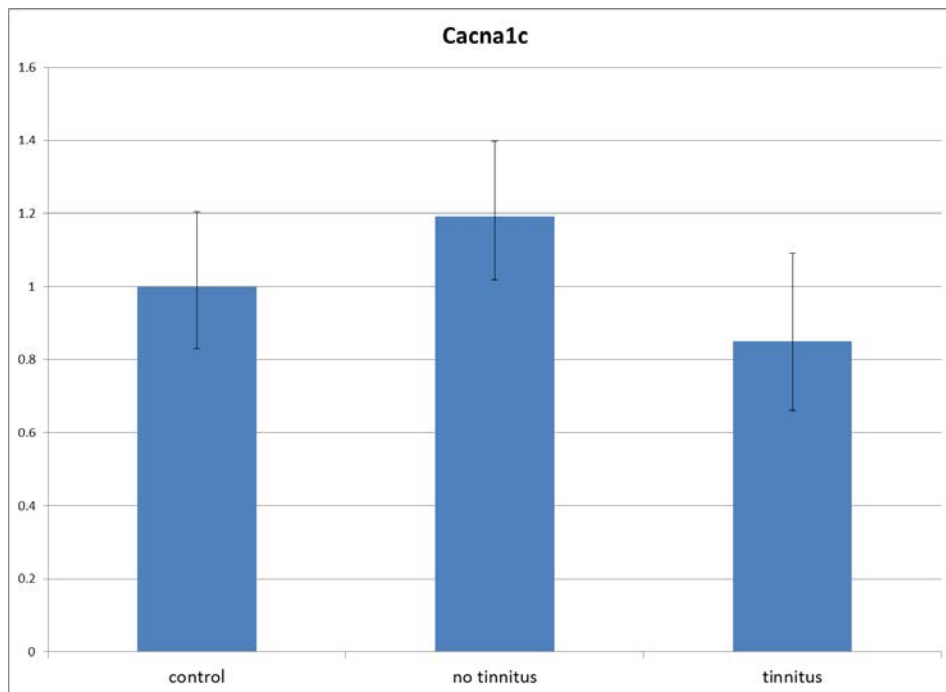
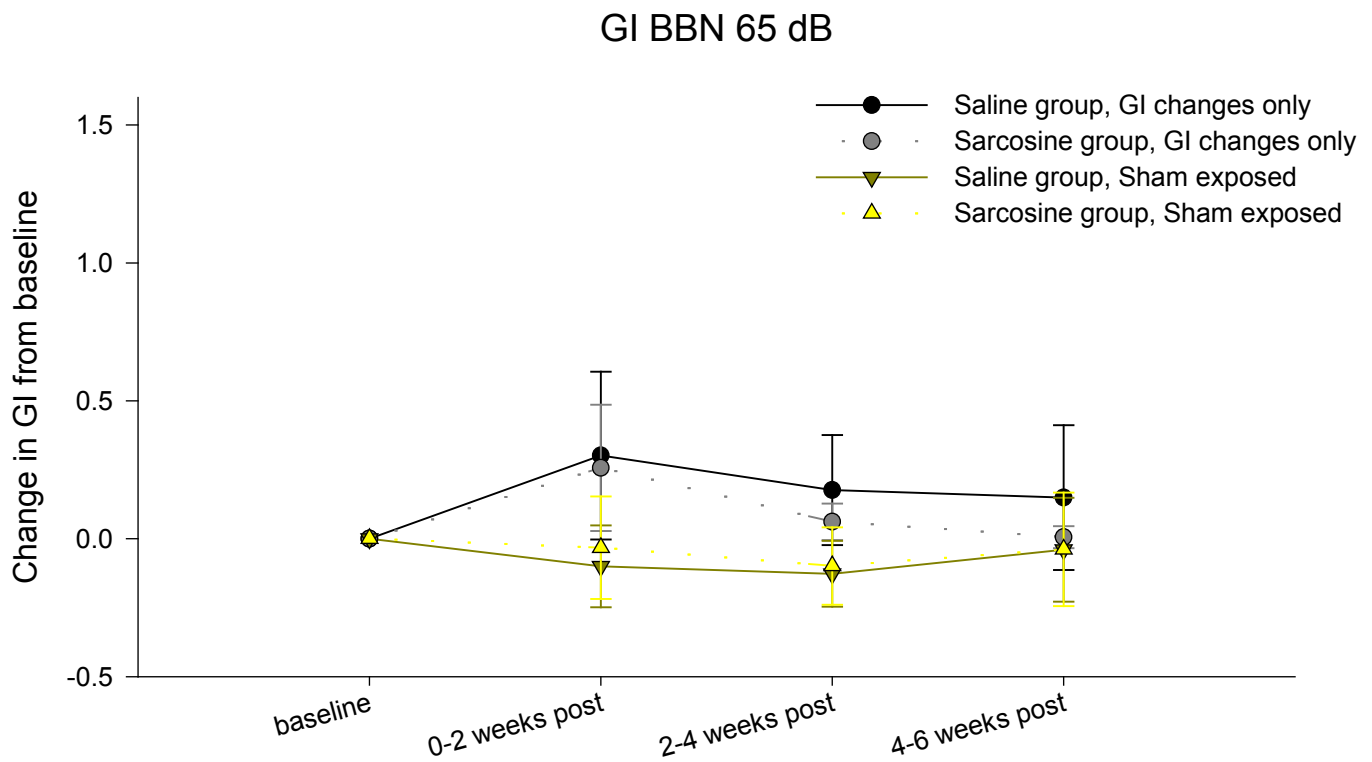


FIGURE NINE – Changes in Gap Inhibition over six week weeks following noise exposure in four groups, two with Sham Noise (one with sarcosine given over the six week period, one with just saline) and two with the small arms fire-like noise (one with sarcosine given over the six week period, one with just saline). Sham groups did not show changes. There was comparable decline in Gap Detection in both noise exposed groups during the first two week, some divergence during the next two weeks and during weeks 4-6 the noise exposed group with sarcosine treatment was comparable to the shams, while the noise exposed without treatment remained reduced.



ABSTRACTS

Karin Halsey; David Dolan; Josef Miller; Susan Shore; Jennifer Eberle; Ariane Kanicki; Susan DeRemer; Diane Prieskorn; Richard Altschuler (2014) Impulse Noise Effects on ABR, Pre-Pulse Inhibition, Gap Detection, and Auditory Nerve Connections, Abstracts, Association for Research in Otolaryngology.

Background: Tinnitus due to noise over-exposure is a condition that affects a significant proportion of the population, especially people exposed to damaging noise, impulse noise, or blast events.

Methods: N = 74 Sprague Dawley rats were unilaterally exposed to impulse noise designed to simulate small arms fire, and compared to matched sham-exposed animals (N=18). In life assessments included Auditory Brainstem Responses (ABRs), Pre-Pulse inhibition (PPI) and Gap inhibition (GI) of the acoustic startle reflex. After perfusion and fixation there was assessment for hair cell loss and loss of CTBP2 immunostaining, a marker for Inner Hair Cell (IHC) auditory nerve connections.

Results: The sham noise exposure group had no change in any of the in-life assessments. Impulse noise-exposed animals had variable final ABR threshold shifts ranging from none to 65 dB, and many (but not all) showed changes in GI that could be interpreted as evidence of tinnitus. Care was taken to exclude data from any animals with an ABR loss in the unexposed ear, as correlations were found between unexposed ear ABR losses and GI performance. A correlation was found between reduced PPI performance and a noise-induced reduction in CTBP2 immunolabeled IHC - auditory nerve connections. Hair cell loss, ABR I/O and latency results will also be discussed.

Conclusions: Impulse noise resulted in physiological and morphological changes in rats. Reductions in CTBP2 staining had correlations to performance on physiological assessments, especially in PPI. Bilateral hearing loss should be avoided in GI experimental designs.

ABSTRACTS

Dave Dolan, Susan Shore, Josef Miller, Karin Halsey, Diane Prieskorn, Ariane Kanicki, Peter Ghisleni, Rick Altschuler (2013) Simulated Small Arms Fire Exposure in Rats Results in Reduced Gap Inhibition of the Acoustic Startle Reflex Suggesting Induction of Tinnitus, Abstracts, Association for Research in Otolaryngology, 2013.

Background:

Hearing loss resulting from exposure to ammunition discharge is a common finding in our military population. Both hearing loss and tinnitus are the leading causes for disability discharge from military service. Prevention or reduction of hearing loss and tinnitus would be of great social and economic benefit. Here we present preliminary findings of an animal model using simulated small arms fire (SAR) to induce hearing loss and tinnitus.

Methods:

Sprague Dawley rats were unilaterally exposed to 50 biphasic impulses via a compression driver over a 2.5 minute period. The impulse level was either 152 or 160 dB SPL. Control rats received sham noise exposure. ABR measurements evaluated the effect of the exposure. The acoustic startle response (ASR), pre-pulse (PPI) and gap inhibition (GI) were used to evaluate the development of tinnitus.

Results:

Sham treated animals showed stable ABR thresholds and consistent ASR, PPI and GI throughout the experiment. In general, animals exposed to the 160 dB stimulus showed significant unilateral ABR threshold shifts and reduced startle amplitudes sometimes resulting in “floor” effects and inconclusive PPI and GI ASR results. Animals exposed to the 152 dB stimulus showed reduced elevations in ABR thresholds compared to the 160 dB exposure. Startle amplitudes were either unaffected by the 152 dB exposure or increased in amplitude. PPI remained intact post exposure. In over half of the animals receiving the 152 dB stimulus GI decreased, suggesting the presence of tinnitus. In some animals, the presence of the gap in noise caused enhancement of the startle response, suggestive of the presence of hyperacusis.

Conclusion:

More than half of the rats exposed to SAR exhibited reduced GI of the ASR but intact PPI, indicative of the induction of tinnitus by this exposure condition.

ABSTRACTS

David Dolan, Karin Halsey, Peter Ghisleni, Richard Altschuler (2012) Effects of Background Noise on the Acoustic Startle Response, Abstracts, Association for Research in Otolaryngology, 2012.

The acoustic startle response (ASR) is a reflex used in human and animal studies of disease. Preceding the startle-inducing stimulus with a lower level acoustic stimulus can reduce the amplitude of the ASR. This effect is called prepulse inhibition (PPI). PPI is considered to be an indicator of sensorimotor gating and is used in metrics of human disorders such as schizophrenia and autism. In animal models using ASR, some studies acquire the startle response in the presence of a background noise. In this case, the “pre-pulse” is a brief increase in the noise amplitude. Here we describe the effects of nine conditions of background noise and their influence on ASR amplitude input/output functions compared to the ASR acquired in quiet. The noise conditions include broadband noise, and 2-kHz bandwidth noise centered at various frequencies (mice 12-14 kHz and 24-26 kHz, and rats 8-10 kHz and 16-18 kHz). These responses were measured in rats (Sprague Dawley) and 2 strains of mice (UMHET4 and CBA/J). In general, the effect of any of the background noise conditions has little or no effect on the ASR amplitude obtained from rats. In contrast, the effect of broad-band noise on the ASR amplitude obtained from mice significantly increases with increases in background noise from 40 to 75 dB SPL. In some cases, mice exhibited ASR reduction in the presence of narrow-band noise. These results suggest that studies using pre-pulse inhibition of the ASR should be aware that the amplitude of the ASR can vary with the acoustic environment and needs to be controlled.